THE PREPARATION OF MEDIA USED IN THE PRESENCE – ABSENCE PROCEDURE

Ontario

Ministry of the Environment

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7.) Preparation of Media Used in Presence-Absence (P-A) Procedure

I) MacConkey Broth (with Tryptone) - Presumptive (P-A Test) Medium

When parallel membrane filter and presence-absence (P-A) tests are conducted the size of bottle used is referred to as the 6 oz (175 ml) P-A bottle. This size bottle is prepared to contain a final volume of 50 ml of medium after autoclaving. At a later time, 50 ml of sample will be added to this bottle.

When drinking water samples receive only the P-A test, the size of bottle used is referred to as the 8 oz (250 ml) P-A bottle. This size bottle is prepared to contain a final volume of 75 ml of medium after autoclaving, to which 100 ml of sample are added subsequently.

Because of similarities in preparation of the medium for 6 and 8 oz P-A bottles, one set of instructions will be given with any differences being clearly indicated.

a) Apparatus required:

1) stirring hot plate

2) 7 litre stainless steel beaker

3) stirring magnet

4) asbestos gloves

5) top load balance, sensitive to 0.1 g

6) spatula

7) automatic dispenser set at 26 ml

8) 4 racks for 6 oz bottles or 4 racks for 8 oz bottles

9) 120 - 6 oz bottles plus rubber-lined bakelite caps or 95 - 8 oz bottles plus rubber-lined bakelite caps

10) 95 or 120, 12 x 75 mm test tubes

11) 1 or 2 litre graduated cylinder

12) weighing dish

b) Reagents required:

1) dehydrated MacConkey Broth (BBL)

2) Tryptone (Difco) or Trypticase (BBL)

3) distilled water

c) Preparation of 6 litres of medium for 6 oz P-A bottles (or 7 litres of medium for 8 oz P-A bottles):

1) Measure out 6 litres of distilled water into the stainless steel beaker; place the stirring magnet (alcohol flamed) in the beaker; put the beaker on the stirring hot plate and activate the stirring mechanism to medium speed (without use of heat).

2) Check the balance level and zero the balance; place the weighing dish on the balance and tare the balance to zero.

3) Using a clean spatula, carefully weigh out 60.0 g of dehydrated Tryptone powder into the weighing dish (77.0 g for 8 oz bottles). Pour the Tryptone powder slowly into the distilled water which is being stirred in the stainless steel beaker.

4) Using a clean spatula, carefully weigh out 420 g of the dehydrated MacConkey Broth medium (544.0 g for 8 oz bottles).

5) When the Tryptone has dissolved, slowly add the MacConkey Broth powder into the stainless steel beaker, while continuing to stir the Tryptone-water medium.

6) During the above procedure, the 12 x 75 mm test tubes should be added to the 6 or 8 oz bottles. One tube should be placed in inverted position in each bottle.

7) The medium is kept stirring continuously until all the MacConkey Broth powder has gone into solution (about 10 minutes). At this time, the medium for use in the 8 oz P-A bottles has one litre of distilled water added to make the final volume up to 7 litres.

8) The dissolved medium is moved to the dispensing area and the automatic dispenser is checked to determine that the dispensed volume of medium per cycle is 26 ml. The medium is now dispensed at the rate of two 26 ml volumes per 6 oz bottle and three 26 ml volumes per 8 oz bottle.

9) The bottle caps are placed on the bottles in a loosened position and two racks of bottles are placed in each autoclave for a period of 5 - 10 minutes. This allows for preheating or warming of the bottles and medium before beginning the autoclave cycle.

10) The autoclave period is 12 minutes at 121°C. The racks of bottles should be removed from each autoclave as soon as the pressure in the chamber returns to zero. The total time in the autoclave following the start of the cycle should not exceed 30 minutes.

11) The racks of bottles should be placed in the refrigerator within 10 minutes after they are removed from the autoclave. When the bottles have cooled after several hours, the caps should be tightened and the bottles may be removed from the refrigerator and stored at room temperature for up to one month.

12) The final pH of the medium is 7.3 ± 0.2 at $25^{\circ}C$.

II) EC Medium - this medium is used in the confirmatory part of the P-A test scheme for both coliforms and fecal coliforms.

a) Apparatus required:

1) stirring hot plate

2) 2 litre stainless steel baker

3) stirring magnet

4) asbestos gloves

5) top load balance, sensitive to 0.1 g

6) spatula

7) automatic dispenser set at 10 ml

8) 16 x 150 mm test tubes (100 tubes)

9) 8 x 45 mm test tubes (100 tubes)

10) 16 mm metal or plastic caps (100 caps)

11) 3 test tube baskets

12) 1 or 2 litre graduated cylinder

13) weighing dish

b) Reagents required:

1) EC Medium (Difco)

2) distilled water

c) Preparation of 1 litre of EC Medium:

1) Measure out 1 litre of distilled water and pour into the 2 litre beaker.

2) Place a stirring magnet (alcohol flamed) in the beaker and place the beaker on the stirring hot plate. Activate the stirrer to medium speed (without use of heat).

3) Check balance level and zero the balance. Place the weighing dish on the balance and tare the balance to zero.

4) Weigh out 37.0 g of EC Medium powder into the weighing dish with a clean spatula.

5) Slowly pour the EC powder into the litre of distilled water and continue the stirring action until all the powder is in solution.

6) Place in each 16 x 150 mm test tube, a single inverted 8 x 45 mm test tube.

7) When the EC Medium is in solution, place the beaker next to the automatic pipetting machine and check to see that each cycle of the machine delivers 10 ml.

8) Dispense 10 ml of the medium into each test tube and then place caps on each of the tubes.

9) Autoclave the baskets containing tubes of EC Medium at 121°C for 15 minutes. Remove the baskets from the autoclave as soon as possible after the end of the autoclave cycle.

10) Within 10 minutes, store the medium in the refrigerator for no longer than two weeks before use.

11) Final pH of the medium is 6.9 ± 0.2 at 25°C.

III) Drake's Medium - this medium is used to determine the presence of fluorescent pseudomonads, particularly Pseudomonas aeruginosa. If the medium is used in a replicate tube dilution series, the most probable number (MPN) of fluorescent pseudomonads may be determined.

a) Apparatus required: similar to that required for the EC Medium except for:

1) 18 x 150 mm test tubes (100 tubes)

2) 18 mm metal or plastic caps (100 caps)

3) 8 x 45 mm tubes are not required

b) Reagents required:

1) Magnesium sulphate ($MgSO_4.7H_2O$) Fisher

2) Potassium sulphate (K_2SO_4) Anachemia

3) Di-potassium hydrogen phosphate (K_2HPO_4) BDH

4) l-asparagine - BDH

5) Glycerol - Analar

6) Distilled water

c) Preparation of 1 litre of Drake's Medium:

1) Put 1 litre of distilled water into a 2 litre stainless steel beaker. Place a stirring magnet (alcohol flamed) in the beaker and place it on a stirring hot plate. Activate the stirrer to medium speed (without use of heat).

2) Check the balance level and zero the balance. Place a weighing dish on the balance and tare the balance to zero.

3) Weigh out separately and dissolve individually in the litre of distilled water, each of the following ingredients:

a) 0.5 g magnesium sulphate

b) 10.0 g potassium sulphate

c) 1.0 g di-potassium hydrogen sulphate

d) 10.0 g glycerol (may be more conveniently weighed out into 100 ml beaker than a weighing dish)

4) When the ingredients have dissolved, dispense 10 ml of the medium into each test tube, cap the tubes and autoclave for 15 minutes at 121°C.

5) Remove the baskets of tubes from the autoclave at the end of the autoclave cycle and refrigerate them within 10 minutes for no longer than two weeks.

6) Final pH of the medium is 7.6 ± 0.2 at $25^{\circ}C$.

IV) Ethyl Violet Azide Broth (EVA) - this medium provides a confirmation test for the presence of fecal streptococci.

a) Apparatus required: similar to that for the EC Medium except that no 8 x 45 mm gas tubes are required

b) Reagents required:

1) Ethyl Violet Azide (EVA) Broth (BBL)

2) distilled water

c) Preparation of 1 litre of EVA Broth

1) Measure out 1 litre of distilled water into the two litre stainless steel beaker. Place a stirring magnet (alcohol flamed) in the beaker and place it on the stirring hot plate. Activate the stirrer to medium speed (without use of heat).

2) Check the balance level and zero the balance. Place a weighing dish on the balance and tare the balance to zero.

3) Weigh out 35.8 g of the EVA powder into the weighing dish with a clean spatula. Slowly pour the EVA powder into the litre of distilled water and continue the stirring action until all the powder has dissolved.

4) When the ingredients have dissolved, dispense 10 ml of the medium into each test tube using the automatic pipetting machine.

5) Cap the tubes and autoclave the medium for 15 minutes at 121°C. Remove the baskets of tubes from the autoclave as soon as the cycle is finished and refrigerate them within 10 minutes for no longer than two weeks before using.

6) Final pH of the medium is 7.0 $\pm$ 0.2 at 25°C.

V) Skim Milk Broth - this medium determines the presence of Clostridium perfringens, which gives a "stormy fermentation" reaction in the clotted milk. Anaerobic conditions must be established in the medium prior to use.

a) Apparatus required:

1) stirring hot plate

2) 2 litre stainless steel beaker

3) stirring magnet

4) asbestos gloves

5) top load balance, sensitive to 0.1 g

6) spatula

7) automatic dispenser set at 10 ml

8) 18 x 150 mm test tubes (50 tubes)

9) 18 mm metal or plastic caps (50 caps)

10) iron nails - 1" long (50 nails)

11) 2 test tube baskets

12) 1 or 2 litre graduated cylinder

13) weighing dish

b) Reagents required:

1) Skim Milk (Difco)

2) distilled water

c) Preparation of 1 litre of Skim Milk Broth:

1) The iron nails should be soaked in xylene for 24 hours; rinsed several times in distilled water to remove all of the xylene; and placed in an oven overnight to dry them off. One nail should be placed in each test tube.

2) Put 1 litre of distilled water into the stainless steel beaker. Place a stirring magnet in the beaker and place it on a stirring hot plate. Activate the stirring mechanism to medium speed (without use of heat).

3) Check the balance level and zero the balance. Place a weighing dish on the balance and tare the balance to zero.

4) Weigh out 100 g of the Skim Milk powder into the weighing dish using a clean spatula.

5) Using the spatula, gradually add the Skim Milk powder to the distilled water and continue the stirring action until all of the powder has dissolved.

6) Using the automatic dispenser, two 10 ml volumes of the Skim Milk broth are dispensed into each test tube to give a final volume of 20 ml.

7) The tubes are capped and the medium is autoclaved at 121°C for 12 minutes. The baskets of tubes are removed quickly at the end of the autoclave cycle and placed in a refrigerator within ten minutes. The tubes should be used within two weeks and the medium has to be boiled before use to achieve anaerobic conditions suitable for C. perfringens. The final pH is 6.4 ± 0.2 at 25°C.

VI) Tryptophane Broth - this medium is used for determining whether indole production takes place following inoculation with a pure culture of an organism.

a) Apparatus required:

1) stirring hot plate
2) 1 litre stainless steel beaker
3) stirring magnet
4) asbestos gloves
5) top load balance, sensitive to 0.1 g
6) spatula
7) automatic dispenser set at 5 ml
8) 13 x 100 mm test tubes (100 tubes)
9) 13 mm metal or plastic caps (100 caps)
10) 3 test tube baskets
11) 1 litre graduated cylinder

b) Reagents required:

1) Tryptone (Difco) or Trypticase (BBL)
2) sodium chloride (NaCl) BDH
3) distilled water

c) Preparation of 1/2 litre of Tryptophane Broth

1) Put 500 ml of distilled water into the stainless steel litre beaker. Place a stirring magnet in the beaker and place it on the stirring hot plate. Activate the stirring mechanism to run at medium speed (without use of heat).

2) Check the balance level and zero the balance. Place the weighing dish on the balance and tare the balance to zero.

3) Weigh out 5 g of tryptone powder with a clean spatula. Slowly pour the tryptone powder into the 500 ml of distilled water. Weigh out 2.5 g of sodium chloride and add slowly to the contents of the beaker. Continue the stirring of the medium until the tryptone powder is in solution and then dispense into the test tubes in 5 ml volumes.

4) Cap the tubes and place the baskets of media in the autoclave. Sterilize the medium for 15 minutes at 121°C. Remove the baskets at the end of the autoclave cycle and refrigerate within 10 minutes. The medium should be used within a two-week period.

5) The final pH of the medium is 7.0 ± 0.2.

VII) Acetamide Agar slants - this medium is used to demonstrate the presence of Pseudomonas aeruginosa.

a) Apparatus required:

1) stirring hot plate
2) 2 litre stainless steel beaker
3) stirring magnet
4) asbestos gloves
5) top load balance, sensitive to 0.1 g
6) spatula
7) automatic dispenser set at 10 ml
8) screw cap tubes 20 x 125 mm (100 tubes)
9) rubber lined, bakelite screw caps 20 mm size (100 caps)
10) 4 test tube baskets
11) 6 slanting racks
12) analytical balance, sensitive to 0.0001 g
13) pH meter
14) thermometer (0-110°C)
15) aluminum foil
16) 1 litre graduated cylinder

b) Reagents required:

1) Sodium Chloride (NaCl) BDH
2) Di-potassium hydrogen phosphate (K_2HPO_4) BDH
3) Acetamide (CH_3CONH_2) J.T. Baker Chem. Co.
4) Potassium dihydrogen phosphate (KH_2PO_4) Anachemia
5) Magnesium sulphate ($MgSO_4.7H_2O$) Fisher Scientific
6) Phenol Red Indicator - Matheson, Coleman and Bell

7) Agar - Difco

8) Distilled water

c) Preparation of 1 litre of Acetamide Agar slants:

1) Measure out 1 litre of distilled water into the 2 litre stainless steel beaker. Place the stirring magnet in the beaker and put it on a stirring hot plate, activating the stirring mechanism to medium speed.

2) Check the balance level and zero the balance. Place the weighing dish on the balance and tare the balance to zero.

3) Weigh out individually the following ingredients and dissolve consecutively in the distilled water:

a) sodium chloride 5.0 g

b) di-potassium hydrogen phosphate 1.4 g

c) acetamide 10.0 g

d) potassium dihydrogen phosphate 0.7 g

e) magnesium sulphate 1.0 g

f) phenol red indicator 0.012 g (use analytical balance)

4) When the ingredients are in solution, the pH should be checked and adjusted to pH 6.8 with 0.1 N NaOH or HCl as required.

5) Weigh out 15.0 g of agar and add slowly to the beaker. The mouth of the beaker is then covered with aluminum foil and a thermometer is inserted through the aluminum foil. Heat is applied to the beaker until the medium starts to boil or the agar shows signs of being in solution about 90-92°C.

6) The medium is then dispensed in 5.0 ml volumes into the tubes; the tubes are loosely capped; and the medium is autoclaved for 15 minutes at 121°C.

7) The baskets of tubes are removed from the autoclave at the end of the cycle and the tubes are placed individually on slanting racks which are slanted to provide a butt about 20 mm deep.

8) When the medium has cooled and solidified, the caps are tightened and the medium is placed in the refrigerator for a period not exceeding 3 months. The final pH of the medium is 6.8 ± 0.2.

VIII) MacConkey Agar plates - this medium is used for isolation of Gram negative bacteria and is particularly useful for determining lactose reactions of coliform bacteria.

a) Apparatus required:

1) 2 stirring hot plates

2) 2 litre stainless steel beaker

3) stirring magnet

4) asbestos gloves

5) top load balance, sensitive to 0.1 g

6) spatula

7) 1 litre graduated cylinder

8) weighing dish

9) thermometer (0-110^{o}C)

10) 100 x 15 mm square sterile plastic petri plates (40 plates)

11) 2 plastic cakettes with lids

12) 500 ml sterile beaker

13) aluminum foil

14) electric fan

15) 5% Wescodyne

16) sterile 500 ml beaker

b) Reagents required:

1) MacConkey agar (Difco)

2) distilled water

c) Preparation of 1 litre of MacConkey Agar medium

1) Measure out 1 litre of distilled water into the stainless steel beaker. Place a stirring magnet (alcohol flamed) in the beaker and put it on a stirring hot plate, which has the stirring mechanism set for medium speed.

2) Check the balance level and zero the balance. Place a weighing dish on the balance and tare the balance to zero.

3) Using the spatula, weigh out 50.0 g of the MacConkey Agar powder and slowly add it to the distilled water being stirred in the beaker. Cover the mouth of the beaker with aluminum foil and insert a thermometer through the foil and attach the thermometer to the side of the beaker. Heat the medium to a slow boil or until the agar medium has gone into solution (90-92°C).

4) Remove the thermometer and place the beaker of dissolved medium in the autoclave for a period of 15 minutes at 121°C. At the end of the autoclave cycle, using asbestos gloves, place the beaker of sterilized medium on a "cold" stirring hot plate.

5) Set the stirring mechanism to medium speed and position the electric fan beside the beaker. Turn on the fan to assist with cooling the medium to about 50-55°C. A thermometer which has been kept in 95% ethyl alcohol is removed from the alcohol and allowed to drip dry. The thermometer with a clip attachment is fastened in an aseptic manner inside the beaker (through the aluminum foil) until the temperature has cooled sufficiently for pouring plates.

6) During the cooling period, the bench of the laminar flow hood is swabbed with Wescodyne disinfectant and the petri dishes are laid out in four or five rows.

7) When the MacConkey medium has reached 50-55°C, the thermometer is removed and the sterile 500 ml beaker is 3/4 filled with the medium. The 500 ml beaker provides a more convenient device for pouring plates than the large litre beaker.

8) Using aseptic procedure, about 25 ml of the medium are poured into each petri dish. The lids are left slightly open (about 10%) to facilitate solidification of the medium and avoid excess condensation of moisture on the lids of the petri plates. After the medium has solidified, the lids are closed and the petri dishes are inverted and left overnight on a bench to allow drying of the surface of the medium.

9) The following day, the petri dishes, still in an inverted position, are placed in plastic cakettes and stored in the refrigerator for use within 4 weeks. The final pH of the medium is 7.1 ± 0.2.

IX) Enterococcus Agar plates - this medium provides a confirmatory test for the presence of fecal streptococci isolated from the presumptive positive P-A bottles.

a) Apparatus required:

1) 2 stirring hot plates

2) 2 litre sterile Erlenmeyer flask

3) 1 litre sterile graduated cylinder

4) 1 sterile 500 ml beaker

5) stirring magnet

6) asbestos gloves

7) top load balance, sensitive to 0.1 g

8) spatula

9) thermometer (0-110^{o}C)

10) large forceps

11) 100 x 15 mm square sterile plastic petri dishes (40 plates)

12) 2 plastic cakettes with lids

13) aluminum foil

14) electric fan

15) 5% Wescodyne

b) Reagents required:

1) m-Enterococcus Agar (Difco)

2) 1 litre sterile distilled water in Erlenmeyer flask

3) 95% ethyl alcohol

c) Preparation of 1 litre of m-Enterococcus Agar medium

1) Check the balance level and zero the balance. Place the sterile 2 litre Erlenmeyer flask on the balance and tare the balance to zero.

2) Using a spatula which has been previously dipped in alcohol and flamed, weigh out 42.0 g of the m-Enterococcus Agar powder directly into the sterile flask. Similarly, dip a stirring magnet into 95% alcohol, flame it and transfer it to the sterile flask using a pair of large forceps. (Make sure the flame is out, before placing the magnetic stirrer in the flask.)

3) Place the flask on a stirring hot plate and add about half of the litre of sterile distilled water. Activate the stirring mechanism to medium speed to promote the mixing of the water and powder and then slowly add the remainder of the litre of water.

4) Check to see that none of the medium is stuck to the bottom of the flask and then turn on the heating element to raise the temperature to 92°C, while continuing the stirring action of the medium. An aluminum foil cover should be present over the top of the flask during the entire procedure and a thermometer previously swabbed with 95% alcohol should be attached to the side of the flask during the heating period.

5) As the temperature reaches 92°C, bubbles will form indicating the medium is on the verge of boiling. The heat should be turned off at this point, but the flask should be left on the hot plate while continuing the stirring action until it is ascertained that the medium is a clear, straw colour indicating the agar has gone into solution.

6) The flask is then transferred to a "cold" stirring hot plate, which has the stirring mechanism activated to medium speed. The electric fan is moved into position and turned on to assist in cooling the medium quickly.

7) During the cooling period, the bench of the laminar flow hood is swabbed with the 5% Wescodyne disinfectant and the petri dishes are laid out in 4 or 5 rows.

8) When the medium had reached 50-55°C, the thermometer is removed and the sterile 500 ml beaker is 3/4 filled with the medium for the purpose of obtaining a convenient device for pouring plates.

9) Using aseptic procedure, about 25 ml of the medium are poured into each petri dish. The lids are left slightly open (about 10%) to facilitate solidification of the medium and avoid excess moisture condensation on the petri dish lids. After the medium has solidified, the lids are closed and the petri dishes are inverted and left overnight on a bench to allow drying of the surface of the medium.

10) The following day, the petri dishes are placed in the plastic cakettes in an inverted position and stored in the refrigerator for use within two weeks. The final pH of the medium is 7.2 ± 0.2.

X) Nutrient Gelatin Agar plate - this medium is used for determining which organisms produce significant gelatinase within a 48 hour period. Also inoculum from this medium is used to test for oxidase production.

a) Apparatus required:

1) 2 stirring hot plates

2) 2 - 1 litre stainless steel beakers

3) stirring magnet

4) asbestos gloves

5) top load balance, sensitive to 0.1 g

6) spatula

7) 1 litre graduated cylinder

8) weighing dish

9) thermometer (0-110^{o}C)

10) 100 x 15 mm round, glass or plastic petri plates (50 plates)

11) 2 plastic cakettes with lids

12) aluminum foil

13) electric fan

14) sterile 500 ml glass beaker

15) 5% Wescodyne

b) Reagents required:

1) Nutrient Agar (Difco)

2) Gelatin (Difco)

3) Yeast Extract (Oxoid)

4) distilled water

c) Preparation of 1 litre of Nutrient Gelatin Agar medium

1) Measure out 500 ml of distilled water into a stainless steel beaker. Place a stirring magnet (alcohol flamed) in the beaker and put it on a stirring hot plate which has the stirring mechanism set for medium speed.

2) Check the balance level and zero the balance. Place a weighing dish on the balance and tare the balance to zero.

3) Using the spatula, weigh out 23 g of Nutrient agar powder into the weighing dish and slowly add to the contents of the beaker. Turn on the heating element; fasten a thermometer to the inside of the beaker; and bring the temperature up to about 30-40°C.

4) Weigh out 3.0 g of Yeast Extract and add it to the Nutrient Agar medium.

5) Weigh out 30.0 g of gelatin powder into a second stainless steel beaker containing 500 ml of distilled water. Place on a stirring hot plate, activate the stirring mechanism and the heating element until the temperature reaches 30-40°C and the gelatin has dissolved. Add the gelatin solution to the beaker containing the Nutrient Agar and Yeast Extract.

6) Cover the mouth of the beaker with aluminum foil and with the thermometer still in place continue heating the medium until the temperature has reached 90-92°C. When the medium has a clear appearance, indicating that all the ingredients have dissolved, the thermometer is removed, fresh aluminum foil is placed over the mouth of the beaker, which is then transferred to the autoclave.

7) The autoclaving time is 15 minutes at 121°C. The beaker is removed from the autoclave at the end of the cycle using asbestos gloves and placed on a "cold" stirring hot plate. An alcohol-disinfected thermometer is again inserted into the beaker; the stirring mechanism is set at medium speed; the electric fan is turned on beside the beaker and the temperature is allowed to drop to 50-55°C.

8) During the cooling period, the bench of the laminar flow unit is swabbed with 5% Wescodyne and the petri plates are laid out in four to five rows.

9) When the temperature has cooled sufficiently for pouring, the thermometer is removed and the sterile 500 ml beaker is 3/4 filled with the sterile medium. This beaker is then used for pouring about 20 ml of medium into each petri dish. The lids are left slightly open (about 10%) to facilitate cooling of the medium and avoid excess condensation on the lids of the petri dishes. When the medium has cooled and solidified, the lids are closed and the petri dishes are inverted and left overnight on the bench to allow drying of the surface of the medium.

10) The following day, the petri dishes are placed in the cakettes in an inverted position and stored in the refrigerator for use within two weeks. The final pH of the medium is 6.8 ± 0.2.

XI) Skim Milk Agar plates - this medium is used for determining a number of the characteristics associated with Pseudomonas aeruginosa such as pigmentation, caseinase production, fluorescence, and oxidase reaction.

a) Apparatus required:

1) 2 stirring hot plates
2) 2 litre stainless steel beaker
3) 1 litre stainless steel beaker
4) 2 stirring magnets
5) asbestos gloves
6) top load balance, sensitive to 0.1 g
7) spatula
8) 1 litre graduated cylinder
9) weighing dish
10) thermometer (0-110°C)
11) 100 x 15 mm round or square plastic petri dishes (40 dishes)
12) 2 plastic cakettes with lids
13) aluminum foil
14) electric fan
15) sterile 500 ml glass beaker
16) 5% Wescodyne

b) Reagents required:

1) dehydrated Skim Milk powder
2) Agar (Difco)
3) Distilled water

c) Preparation of 1 litre of Skim Milk Agar plates

1) Measure out 500 ml of distilled water into the 2 litre beaker. Place a stirring magnet (alcohol flamed) in the beaker and put it on a stirring plate which has the stirring mechanism set for medium speed.

2) Check the balance level and zero the balance. Place a weighing dish on the balance and tare the balance to zero.

3) Weigh out 100 g of Skim Milk powder and add it slowly to the 500 ml of distilled water. Allow the mixture to stir without heat for approximately 30 minutes.

4) During this period, measure out 500 ml of distilled water into the 1 litre beaker. Place a stirring magnet (alcohol flamed) in the beaker and put it on a stirring hot plate, which has the stirring mechanism set at medium speed.

5) Using a spatula, weigh out 15.0 g of agar and slowly add the agar to the 500 ml of distilled water. Turn on the heating element, cover the beaker mouth with aluminum foil, place a thermometer on the inside of the beaker, and allow the temperature of the medium to slowly rise to 90-92^{o}C. This usually takes 10-12 minutes.

6) Both the beaker containing the dissolved agar and the beaker containing the dissolved Skim Milk are covered with aluminium foil and placed in the autoclave for sterilization at 121^{o}C for 12 minutes. Both beakers are removed from the autoclave immediately and placed on stirring plates until the temperature of each has cooled to approximately 55^{o}C.

7) At the start of the cooling period, the laminar flow hood is swabbed with 5% Wescodyne and the petri dishes are laid out in four to five rows.

8) The Skim Milk solution is then aseptically added to the agar solution and the mixture is stirred for an additional two to three minutes until the temperature has dropped to 50-52^{o}C.

9) When the temperature has cooled sufficiently for pouring plates, the sterile 500 ml beaker is 3/4 filled with the Skim Milk medium. This beaker is used for pouring about 20-25 ml of medium into each petri dish. The lids are left slightly open to permit cooling of the medium without excess condensation on the lids of the petri dishes. When the medium has solidified, the lids are closed and the petri dishes are inverted. They are then placed in plastic cakettes into the refrigerator for use within two weeks. The final pH of the medium is 6.4 $\pm$ 0.2.

XII) Mannitol Salt Agar plates - this medium is used for the isolation and differentiation of Staphylococcus aureus.

a) Apparatus required:

1) 2 stirring hot plates

2) 2 litre stainless steel beaker

3) stirring magnet

4) asbestos gloves

5) top load balance, sensitive to 0.1 g

6) spatula

7) 1 litre graduated cylinder

8) weighing dish

9) thermometer (0-110°C)

10) 100 x 15 mm round or square plastic petri dishes (40 dishes)

11) 2 plastic cakettes with lids

12) aluminum foil

13) electric fan

14) sterile 500 ml beaker

15) 5% Wescodyne

b) Reagents required:

1) Dehydrated Mannitol Salt Agar (Difco)

2) Distilled water

c) Preparation of 1 litre of Mannitol Salt Agar plates

1) Measure out 1 litre of distilled water into the 2 litre beaker. Place a stirring magnet (alcohol flamed) in the beaker and put it on a stirring hot plate, which has the stirring mechanism activated to medium speed.

2) Check the balance level and zero the balance. Place a weighing dish on the balance and tare the balance to zero.

3) Using a clean spatula, weigh out 111.0 g of the Mannitol Salt Agar powder into the weighing dish. Slowly add the powder to the distilled water stirring in the 2 litre beaker. Turn on the heating element and when the powder has been thoroughly wetted, cover the beaker with aluminum foil, place a thermometer in the beaker and allow the contents of the beaker to heat up to 90-92^{o}C. At this time, the thermometer is removed from the beaker, the hole in the aluminum foil is patched with a small piece of autoclave tape and the beaker is placed in the autoclave for sterilization at 121^{o}C for 15 minutes.

4) At the end of the autoclave cycle, the beaker is removed, using asbestos gloves, and placed on a "cold" stirring hot plate. The stirring mechanism is set at medium speed and a fan is moved into position to assist with cooling the temperature down to 50-55^{o}C. An alcohol-disinfected thermometer is used to periodically check the temperature in the beaker.

5) During the cooling period, the laminar flow unit is switched on for a 30 minute period; the bench of the laminar flow hood is swabbed with 5% Wescodyne and the petri dishes are laid out.

6) When the temperature has cooled sufficiently for pouring plates, the sterile 500 ml beaker is 3/4 filled with the Mannitol Salt Agar. This beaker is used for pouring 20-25 ml of medium into each petri dish. The lids are left slightly open to permit cooling of the medium without excess condensation occurring on the lids of the petri dishes. When the medium has solidified, the lids are closed and the petri dishes are inverted. They are placed in plastic cakettes into the refrigerator for use within two weeks. The final pH of the medium is 7.4 $\pm$ 0.2.

XIII) Arginine Dihydrolase medium - Although this medium is used in the P-A procedure for confirmation of Aeromonas spp., Pseudomonas organisms will also produce a positive test with this medium.

a) Apparatus required:

1) 1 stirring hot plate
2) 2 litre stainless steel beaker
3) stirring magnet
4) top load balance, sensitive to 0.1 g
5) analytical balance, sensitive to 0.0001 g
6) spatula
7) 1 litre graduated cylinder
8) weighing dish
9) thermometer (0-110°C)
10) 10 ml adjustable hand syringe set at 5.0 ml
11) 16 x 100 mm screw cap tubes (200 tubes)
12) 16 mm bakelite screw caps (200 caps)
13) 6 test tube baskets
14) pH meter
15) aluminum foil

b) Reagents required:

1) Peptone (Difco)
2) Sodium Chloride (BDH) NaCl
3) Di-potassium hydrogen phosphate (Anachemia) K_2HPO_4
4) l (+) Arginine hydrochloride (BDH)
5) Phenol red indicator (Matheson, Coleman & Bell)
6) Agar (Difco)
7) Distilled water

c) Preparation of 1 litre of Arginine Dihydrolase medium:

1) Check the balance level and zero the balance. Place a weighing dish on the balance and tare the balance to zero.

2) Using a clean spatula and weighing dish each time, weigh out the following ingredients by the top load balance:

   (a) peptone 1.0 g

   (b) sodium chloride 5.0 g

   (c) dipotassium hydrogen phosphate 0.3 g

   (d) l (+) arginine hydrochloride 10.0 g

   (e) agar 3.0 g

3) Using the analytical balance, weigh out 0.01 g of the phenol red indicator.

4) Measure out 1 litre of distilled water in the 2 litre stainless steel beaker. Place the stirring magnet in the beaker and put it on the stirring hot plate which has the stirring mechanism activated to medium speed.

5) Add each of the ingredients separately into the distilled water, cover the mouth of the beaker with aluminum foil, place a thermometer through the aluminum foil and gradually heat the medium to a temperature of 90-92^{o}C.

6) Remove the beaker from the hot plate and allow the medium to cool to 70^{o}C before checking the pH, which should be 6.8. Any pH adjustments should be made with NaOH or HCl as required.

7) Dispense the hot medium into the tubes in 5.0 ml amounts using the hand syringe. Cap the tubes loosely and then place in the autoclave for 15 minutes at 121^{o}C. Cool the medium in a refrigerator after autoclaving and when the tubes are cooled the caps should be securely tightened. The final pH of the medium is 7.0 $\pm$ 0.1.

XIV) Phenol Réd Dextrose Broth - this medium is used as an additional check on gas production from dextrose fermentation in comparison with the Enterotube result.

a) Apparatus required:

1) stirring hot plate

2) 2 litre stainless steel beaker

3) stirring magnet

4) asbestos gloves

5) top load balance, sensitive to 0.1 g

6) spatula

7) 10 ml adjustable hand syringe or automatic dispenser set at 5.0 ml

8) 13 x 100 mm test tubes (200 tubes)

9) 13 mm metal caps (200 caps)

10) 4 test tube baskets

11) 45 x 7 mm durham tubes (200 tubes)

12) 1 litre graduated cylinder

13) weighing dish

b) Reagents required:

1) Phenol Red Dextrose Broth (Difco)

2) Distilled water

c) Preparation of 1 litre of Phenol Red Dextrose Broth

1) Measure out 1 litre of distilled water into the stainless steel beaker.

2) Put the beaker on the stirring hot plate and activate the stirrer to medium speed.

3) Check the balance level and zero the balance. Place the weighing dish on the balance and tare the balance to zero.

4) Carefully weigh out 21.0 g of the dehydrated Phenol Red Dextrose Broth powder into the weighing dish. Slowly add the powder to the distilled water in the beaker. Continue the stirring action until the powder is dissolved (without use of heat).

5) Place one inverted durham tube in each of the 13 x 100 mm test tubes. Dispense 5 ml of the medium into each of the test tubes using an automatic dispensing device.

6) Place a metal cap on each of the test tubes and place the baskets of tubed medium in the autoclave for 15 minutes at 121°C.

7) Using asbestos gloves, remove the baskets of medium from the autoclave at the end of its cycle and place the baskets in the refrigerator for use within two weeks of preparation.

8) Final pH of the medium is 7.4 ± 0.2 at 25°C.

XV) OF Glucose Medium - this test is used to determine whether the glucose sugar is fermented oxidatively (aerobically) or fermentatively (anaerobically). The medium will assist in differentiation of Staphylococcus from Micrococcus or Aeromonas from Pseudomonas.

a) Apparatus required:

1) 2 stirring hot plate units
2) 2 litre stainless steel beaker
3) 3 stirring magnets
4) asbestos gloves
5) top load balance, sensitive to 0.1 g
6) spatula
7) sterilized 10 ml adjustable hand syringe set at 5.0 ml
8) 16 x 150 mm test tubes (100 tubes)
9) 16 mm metal or plastic caps (100 caps)
10) 3 test tube baskets
11) sterile membrane filter unit (includes funnel, membrane filter holder and receiving flask)
12) sterile membrane filters
13) membrane filter forceps
14) 250 ml sterile Erlenmeyer flask with screw cap
15) 2-250 ml beakers
16) bunsen burner
17) thermometer (0-110^{o}C)
18) 1 litre graduated cylinder
19) 100 ml graduated cylinder
20) weighing dishes
21) pH meter

22) analytical balance, sensitive to 0.0001 g

23) aluminum foil

b) Reagents required:

1) Peptone (Difco)

2) Sodium chloride (NaCl) BDH

3) Di-potassium hydrogen phosphate (K_2HPO_4) Anachemia

4) Agar (Difco)

5) Bromthymol Blue indicator (Difco)

6) d-Glucose (Analar)

7) Distilled water

8) Ethyl alcohol

c) Preparation of 1 litre of OF Glucose medium - this medium requires a number of preparatory steps before ending up with the final product.

1) Sterile test tubes - the tubes are capped and placed in baskets for autoclaving at 121°C for 20 minutes before starting the other parts of the medium preparation.

2) 10% Sterile glucose solution

(i) Check the balance and zero the balance, Place a clean 250 ml beaker on the balance and tare the balance to zero.

(ii) Using a clean spatula previously swabbed with ethyl alcohol, weigh out 10.0 g of d-glucose into the beaker. Place a magnetic stirrer (alcohol flamed) in the beaker. Add 100 ml of distilled water to the beaker. Place the beaker on a cold stirring hot plate and allow to stir at medium speed until the glucose is in solution.

(iii) Set up the sterile membrane filter unit on the sterile receiving flask. Using flamed forceps, place a membrane filter on the holder part of the unit; replace the funnel and slowly pour the glucose solution through the membrane filter. When the glucose solution is filtered into the receiving flask, remove the membrane filter unit and using aseptic technique (flaming mouths of flasks), transfer the contents of the receiving flask to the sterile 250 ml screw-capped Erlenmeyer flask.

3) 0.1% Bromthymol Blue indicator solution

(i) Place 100 ml of distilled water into a 250 ml glass beaker. Add a stirring magnet and activate the stirrer to medium speed.

(ii) Check the analytical balance level and zero the balance. Place a weighing dish on the balance pan and tare the balance to zero. Weigh out 0.1 g of the Bromthymol blue indicator powder. Add the powder to the 100 ml of distilled water and continue stirring until the indicator is in solution.

4) OF base medium

(i) Measure out 900 ml of distilled water into the 2 litre stainless steel beaker. Add a magnetic stirrer and activate the stirring mechanism to medium speed.

(ii) Check the balance level and zero the balance. Place a weighing dish on the balance and tare the balance to zero. Weigh out individually:

a) 2.0 g of peptone

b) 5.0 g of sodium chloride

c) 0.3 g of di-potassium hydrogen phosphate

d) 3.0 g of agar

(iii) Add each of the above ingredients separately to the 2 litre beaker. Cover the mouth of the beaker with aluminum foil, place a thermometer through the aluminum foil into the beaker and turn on the heating portion of the stirring hot plate unit. Heat the medium to almost boiling at 90-92°C while the medium is continually being stirred. Remove the thermometer and add 30 ml of the 0.1% Bromthymol Blue solution.

(iv) Check the pH of the medium and adjust to pH 7.2 if necessary with either 0.1 N HCl or 0.1 N NaOH. Recover the beaker with aluminum foil and place some autoclave tape over the thermometer hole. Autoclave the medium at 121°C for 15 minutes in the 2 litre beaker.

(v) Cool the medium on a cold stirring hot plate to about 75°C. Using a pH probe that has been soaked in 95% alcohol for 5 minutes, check the pH and adjust to pH 7.2 with sterile 0.1N NaOH or 0.1 N HCl, if necessary. When the medium reaches 55-60°C, aseptically add the filter-sterilized 10% glucose solution. Continue the stirring action until the medium reaches about 50°C.

(vi) Unwrap the sterile syringe and feeder tubing, and place the sinker into the 2 litre beaker of OF glucose medium. Keep the aluminum foil cover on the beaker during the pipetting operation.

(vii) While holding the syringe in the right hand, the left hand picks up a sterile capped tube. The cap is removed with the little finger of the right hand; the tip of the syringe is placed in the mouth of the test tube; and two 5 ml amounts of medium are syringed into the tube. The cap is placed back on the test tube, which is placed in a test tube basket. (Usually the first portion of medium brought up into the syringe is wasted because the volume is inaccurate.) Before each syringing into a test tube, the tip of the syringe and the mouth of the test tube are flamed. (Flaming the tip of the syringe alleviates solidification of the medium in the dispensing orfice of the syringe.)

(viii) After the medium has been syringed into all the tubes, they are placed in a refrigerator for use within three weeks of preparation. The syringing apparatus is dismantled and thoroughly washed in hot water to remove all traces of the OF glucose medium.

(ix) Final pH of medium is 7.1 ± 0.1.

XVI) Trypticase Soy Broth - this medium is used for culturing organisms, when an inoculum is required for streaking plates or inoculation of other media or reagents, such as the coagulase test.

a) Apparatus required:

1) stirring hot plate

2) 2 litre stainless steel beaker

3) stirring magnet

4) asbestos gloves

5) top load balance, sensitive to 0.1 g

6) spatula

7) automatic dispenser device set at 5.0 ml

8) 16 x 150 mm test tubes (200 tubes)

9) 16 mm metal or plastic caps (200 caps)

10) 6 test tube baskets

11) 1 or 2 litre graduated cylinder

12) weighing dish

b) Reagents required:

1) Trypticase Soy Broth (BBL)

2) Distilled water

c) Preparation of 1 litre of Trypticase Soy Broth

1) Measure out 1 litre of distilled water and pour into the 2 litre stainless steel beaker.

2) Place a stirring magnet (alcohol flamed) in the beaker and put the beaker on the stirring hot plate. Activate the stirring mechanism to medium speed.

3) Check balance level and zero the balance. Place the weighing dish on the balance and tare the balance to zero.

4) Weigh out 30.0 g of the Trypticase Soy broth powder using a clean spatula into the weighing dish.

5) Slowly pour the powder medium into the beaker of distilled water and continue the stirring action (without heating) until the powder is in solution.

6) Using the automatic dispensing device, add 5.0 ml of the medium to each test tube. Cap the test tubes and place the baskets of test tubes in the autoclave.

7) Autoclave the trypticase soy broth medium at 121^{o}C for 15 minutes. At the end of the autoclave cycle, remove the baskets of test tubes and place in a refrigerator within 10 minutes. Final pH of the medium is 7.3 $\pm$ 0.2.

XVII) Trypticase SoyAgar slants - this is a general purpose medium used for growing bacterial cultures for inoculating other microbiological tests or for storage of cultures for 3-4 weeks.

a) Apparatus required:

1) stirring hot plate

2) 2 litre stainless steel beaker

3) stirring magnet

4) asbestos gloves

5) top load balance, sensitive to 0.1 g

6) spatula

7) automatic dispenser set at 5.0 ml

8) 16 x 100 mm screw cap tubes (200 tubes)

9) 16 mm bakelite screw caps (200 caps)

10) 8 test tube baskets

11) 1 or 2 litre graduated cylinder

12) weighing dish

13) thermometer (0-110^{o}C)

14) aluminum foil

15) 6 slanting racks

b) Reagents required:

1) Trypticase Soy Agar (BBL)

2) Distilled water

c) Preparation of 1 litre of Trypticase Soy Agar

1) Measure out 1 litre of distilled water and pour into the 2 litre stainless steel beaker.

2) Place a stirring magnet (alcohol flamed) in the beaker and put the beaker on the stirring hot plate. Activate the stirring mechanism to medium speed.

3) Check balance level and zero the balance. Place the weighing dish on the balance and tare the balance to zero.

4) Weigh out 40.0 g of the Trypticase Soy agar powder using a clean spatula.

5) Slowly pour the powder medium into the beaker of distilled water. Continue the stirring action and turn on the heating element. Cover the mouth of the beaker with aluminum foil and insert the thermometer through the foil at the side of the beaker. Heat the medium to a slow boil for one minute or until the agar has gone into solution (90-92^{o}C).

6) Remove the thermometer and set up the automatic dispensing device to dispense 5.0 ml of the medium into each test tube. The tubes are loosely capped and the baskets of tubes are placed in the autoclave for 15 minutes at 121^{o}C.

7) The baskets of tubes are removed from the autoclave at the end of the autoclave cycle and when the tubes have cooled sufficiently to be picked up, the tubes are placed individually on the slant racks, which are slanted to provide a butt of about 10 mm long.

8) When the medium has cooled and solidification of the agar has taken place, the caps are tightened and the medium is placed in the refrigerator for a period not exceeding 3 months. Final pH is 7.3 $\pm$ 0.2.